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Physicochemical Conditions and Microbial Activities in the Highly Alkaline Gut of the Humus-Feeding Larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae)

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The soil macrofauna plays an important role in the carbon and nitrogen cycle of terrestrial ecosystems. In order to gain more insight into the role of the intestinal microbiota in transformation and mineralization of organic matter during gut passage, we characterized the physicochemical conditions, microbial activities, and community structure in the gut of our model organism, the humus-feeding larva of the cetoniid beetle *Pachnoda ephippiata*. Microsensor measurements revealed an extreme alkalinity in the midgut, with highest values (pH > 10) between the second and third crown of midgut ceca. Both midgut and hindgut were largely anoxic, but despite the high pH, the redox potential of the midgut content was surprisingly high even in the largest instar. However, reducing conditions prevailed in the hindgut paunch of all instars ($E_h \sim -100$ mV). Both gut compartments possessed a pronounced gut microbiota, with highest numbers in the hindgut, and microbial fermentation products were present in high concentrations. The stimulation of hindgut methanogenesis by exogenous electron donors, such as H_2, formate, and methanol, together with considerable concentrations of formate in midgut and hemolymph, suggests that midgut fermentations are coupled to methanogenesis in the hindgut by an intercompartmental transfer of reducing equivalents via the hemolymph. The results of a cultivation-based enumeration of the major metabolic groups in midgut and hindgut, which yielded high titers of lactogenic, propionigenic, and acetogenic bacteria, are in good agreement not only with the accumulation of microbial fermentation products in the respective compartments but also with the results of a cultivation-independent characterization of the bacterial communities reported in the companion paper (M. Egert, B. Wagner, T. Lemke, A. Brune, and M. W. Friedrich, Appl. Environ. Microbiol. 69:6659–6668, 2003).

The soil macrofauna plays an important role in the carbon and nitrogen cycle of terrestrial ecosystems (18, 41, 62). The intestinal tracts of litter-feeding and humivorous soil macroinvertebrates are favorable habitats for microorganisms and typically harbor a dense and active gut microbiota. The major function commonly attributed to the microorganisms in the guts of such animals is the depolymerization and fermentative breakdown of the cellulosic or lignocellulosic component of their diet, which leads to degradation products that can be resorbed by the host. This is supported by the high concentrations of microbial fermentation products and by the presence of fermentative bacteria and protozoa, accompanied by obligately anaerobic homoacetogenic and methanogenic microorganisms, in the guts of such animals. However, the extent and importance of such processes and their specific function in the nutrition of the host are scarcely understood (for reviews, see references 8, 9, 11, 12, 14, 36, and 44).

In the case of soil-feeding termites, host factors such as the extreme alkalinity of the anterior hindgut and the influx of oxygen seem to play a key role in sequestering organic matter from the inorganic soil matrix (37). The decrease of molecular weight and increase in solubility resulting from alkaline extraction and chemical oxidation render the organic matter accessible for digestion in subsequent, less-alkaline compartments (34, 35). Alkaline gut regions are encountered also in many representatives of other insect orders and seem to be connected with the dietary preferences of the respective taxa (Coleoptera, Diptera, and Lepidoptera; see references 15 and 32). Comparing several beetle larvae feeding on a lignocellulosic diet, Grayson (30) already had pointed out an apparent correlation between the degree of humification of the diet and the alkalinity of the intestinal tract. The highest pH values among beetle larvae were encountered among the Scarabaeidae (50, 52, 58, 60), which comprise species from humivorous, detritivorous, and coprophagous feeding guilds. Although scarab beetle larvae are among those few arthropods that have received at least a minimum of attention from a microbiological perspective (3, 4, 21, 23) and although their importance for the transformation of soil organic matter is undisputed, little is known about the composition of the gut microbial community and its role in the digestive process.

Using the humivorous larva of the cetoniid beetle *Pachnoda ephippiata* (Coleoptera: Scarabaeidae) as a model organism, we investigated the physicochemical conditions and microbial activities in the gut in order to gain more insight into the role of the intestinal microbiota in transformation and mineralization of organic matter during gut passage. Since a representative analysis of the microbial community structure requires covering also those populations that resist cultivation (14), the project also included a cultivation-independent approach, involving molecular cloning and fingerprinting techniques. These

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results are reported and discussed in the companion paper (28).

MATERIALS AND METHODS

Insects. *P. ephippiata* Gerstaecker 1867 was bred in a 100-liter terrarium filled 20 cm deep with soil and illuminated with a 120-W light bulb on a 12-h day-night cycle; the average temperature was around 27°C (22 to 30°C). To ensure a constant high humidity, the walls and the soil surface were sprayed with soft tap water on a daily basis with a hand vaporizer. The terrarium contained freshly cut twigs of *Lonicera nitida* to provide resting places for the beetles. Imagines were fed with fresh fruit (apples, bananas, and peaches) and Chinese lettuce provided in a high-rimmed bowl, so that the food was not accessible to the larvae. The larvae therefore thrived almost exclusively on the substratum, consisting of commercial preparations of formulated organic potting soil, composed of moderately to highly decomposed sphagnum peat, grass compost, and a clay substrate with a high proportion of montmorillonite. Instars (Fig. 1A) were easily differentiated by the diameter of their head capsules. The average fresh weights (minimum-to-maximum range) of the first to third larval instars were 71 (26 to 140) mg (n = 24), 374 (152 to 780) mg (n = 18), and 2,541 (516 to 4,091) mg (n = 48), respectively.

Gut preparation. For dissection, larvae were anesthetized by exposure to a N_2-CO_2 (80/20, vol/vol) gas atmosphere for about 15 min, decapitated with scissors, and fixed with steel pins (ventral side up) in a preparation dish filled with insect Ringer's solution (16). The cuticle was cut along the sidelines, and the ventral integument was removed, the circular muscles and trachea being carefully severed from the skin. After a circular incision around the anus, the intestinal tract was carefully removed from the body; the residual fat body tissue and trachea were removed with a blunt instrument. First- and second-instar larvae were prepared using a dissecting microscope.

For the separate incubation of midgut and hindgut, and for the preparation of gut homogenates, isolated guts were separated at the muscular midgut-hindgut junction into a midgut section, including the short foregut, and a hindgut section, including paunch, colon, and rectum (Fig. 1B to D); no ligation was necessary to prevent leaking of the gut contents. Unless mentioned otherwise, all data reported in this paper are based on the fresh weight of the animal or the respective gut section; dry weight determinations indicated an average water content of approximately 87% for both midgut and hindgut of all instars. Gut volumes were estimated by measuring the outer diameter of the respective gut sections at different points along the axis, with a dissecting scope equipped with an ocular grid, and approximating the shapes to various geometric figures (a combination of truncated cones and cylinders).

The fecal pellets produced by the third instar (when freshly voided) had an average weight of 12 mg and a water content of 50% (calculated from fresh weight and dry weight of 100 fecal pellets). They were produced at a rate of approximately 1.5 pellets h^{-1}. By using the average dry weight of the gut of the third instar (109 mg), it was estimated that a larva produces almost two gut equivalents of feces per day.

CH_4 production rates. Larvae were placed into 10-ml (first and second instar) and 120-ml (third instar) glass vials, which were sealed (under air) with rubber stoppers, and were incubated for several hours at room temperature in the dark. If individual gut compartments were tested, the vials received a small volume of insect Ringer's solution (0.2 or 1 ml [16]) to avoid desiccation of the samples; care was taken not to cover the segments with liquid in order to facilitate gas exchange with the headspace. At regular intervals, gas samples were taken and analyzed for methane by gas chromatography, following the procedure described elsewhere in more detail (53). Stimulation of methane emission was tested by supplementing the headspace with H_2 (20 kPa) or by adding methanol (5 mM) or sodium formate (10 mM) to the Ringer's solution after the basal rate of methane production had been established.

Microsensor measurements. Clark-type oxygen microsensors with guard cathodes (48) were constructed and calibrated as described previously (16). Polarographic hydrogen microsensors had the same principal design (61), except that the working electrode was coated with platinum black (27). Testing and calibration procedures were performed as previously described (27). Both microsensor types had 90% response times of <5 s and tip diameters of 10 to 15 μm. The stirring sensitivity of both electrodes was <1% of the signal obtained upon calibration in air-saturated water or at 20 kPa of H_2, respectively.

Capillary microelectrodes with ion-selective membranes (LIX type) with a 200- to 300-μm-pore-size membrane of hydrogen ionophore I-cocktail A (Fluka) were constructed using the design described by Revsbech and Jørgensen (49). The microsensors were equipped with an external casing filled with 1 M KCl to minimize electrical noise (33) and had tip diameters of 10 to 30 μm and 90% response times of <10 s. The calibration was performed in commercial pH standard solutions (pH 6 to 12) and showed a log-linear response in this range (15). Electrically shielded platinum redox microelectrodes with tip diameters of 10 to 20 μm and response times of 10 to 20 s were constructed and calibrated with freshly prepared quinhydrone solutions as previously described (27). In both cases, the working electrode was connected to a high-impedance electrometer amplifier ($R_i > 10^{14}$ Ω) via a low-noise coaxial cable, and electrode potentials were measured against an Ag-AgCl electrode that was in contact with the

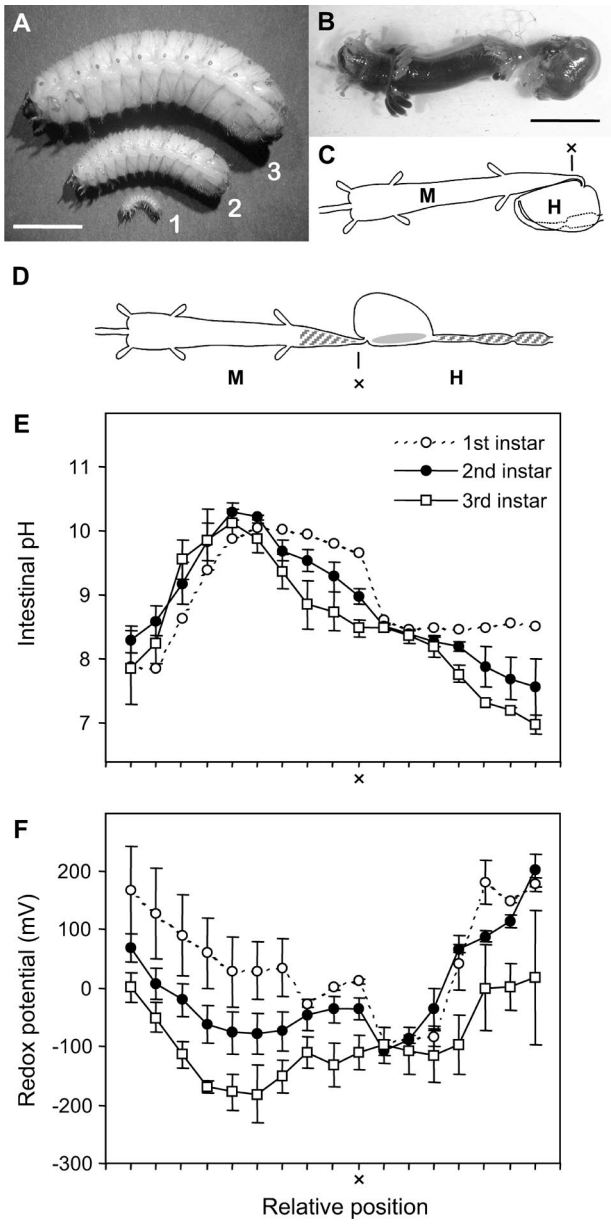


FIG. 1. Habitus of the larval instars (1 to 3) of *P. ephippiata* (A) and of the intestinal tract of the third instar (B and C), showing the three rings of gastric ceca and the point (×) where midgut (M) and hindgut (H) were separated. The gut habitus of the other instars is very similar (not shown). For microsensor measurements, guts were placed fully extended (D) into aerated insect Ringer's solution. Hatching indicates the gut regions of the first instar which were completely penetrated by oxygen, and shading indicates the only region of the hindgut where a slight accumulation of hydrogen was detectable (see text). Axial profiles of intestinal pH (E) and redox potential (F) were determined with microsensors. Bars (A and B), approximately 10 mm.

agarose-filled chamber via a KCl-filled agar bridge (1% agar in 1 M KCl). Reference and casing were grounded.

For the measurements, larvae were dissected, and the intact, fully extended gut (Fig. 1D) was immediately placed into a small polyvinyl chloride chamber (15 by 15 mm; 9 cm long) with glass front and back walls. A 5-mm layer of silicone cast into the bottom of the chamber allowed the gut to be fixed with minute steel pins. The chamber was irrigated with air-saturated insect Ringer's solution (16) by means of a peristaltic pump (5 ml min^{-1}). Measurements were carried out at ambient temperatures (20 to 25°C). Under these conditions, the dissected guts exhibited a moderate peristalsis that persisted for several hours, indicating that they were still physiologically active. Microsensors controlled by a manual micromanipulator were mounted vertically above the chamber; the progress of the tip was observed with a horizontally mounted stereomicroscope. A more detailed description of the setup has been published elsewhere (24).

Total cell counts. Cell numbers were obtained by a standard protocol (46) with the following modifications: gut sections were homogenized and appropriately diluted with phosphate-buffered saline solution and fixed with paraformaldehyde (3%, wt/vol) overnight at 4°C in the dark. Fixed homogenate (1 ml) was filtered onto white polycarbonate membrane filters, washed with phosphate-buffered saline, air dried, and subsequently stained with 4,6-diamino-2-phenylindole (DAPI). Cells were counted by epifluorescence microscopy with an ocular grid; approximately 1,000 to 2,000 cells were counted for each sample.

Metabolites in gut fluid. Individual gut compartments were blotted with tissue paper to remove residual Ringer's solution, transferred into plastic vials containing a defined volume of liquid (see below), and homogenized by using an ultrasonic probe (75 W, 20 s). After centrifugation (15 min at 14,000 × *g*), an aliquot of the supernatant was analyzed using the appropriate method (see below). All procedures for sample preparation were carried out at temperatures between 0 and 4°C.

For the determination of microbial fermentation products, gut sections were homogenized in 100 μl of HCl (400 mM) containing disodium malonate (5 mM) as internal standard, and supernatants were analyzed by high-pressure liquid chromatography (HPLC) (56). For the determination of total dissolved inorganic carbon (ΣCO_2), gut sections were homogenized in 100 μl of NaOH (5 mM), and supernatants were subjected to flow injection analysis (55). With gut sections of second and third instars, the volume of the homogenization fluid was increased to achieve at least a twofold dilution of the gut contents.

Metabolites in hemolymph. Hemolymph was collected from third instars directly after decapitation by using graded 100-μl capillaries; the yield was about 300 to 400 μl per larva. Samples were immediately transferred into the same volume of HCl (200 mM) containing phenylthiourea (2 mM) to prevent clogging (40), centrifuged (15 min at 14,000 × *g*), and analyzed by HPLC (56).

Serial dilution cultures. Midgut and hindgut sections were homogenized separately in sterile anoxic buffered salt solution with glass homogenizers. The homogenates were serially diluted (1:10) in substrate-free medium and inoculated (0.5 ml) into culture tubes containing medium (4.5 ml) amended with the respective substrates; the procedures have been described elsewhere in detail (56).

The medium for anaerobic cultivation was anoxic, bicarbonate-buffered mineral medium (AM-5 [7]), supplemented with yeast extract and Casamino Acids (Difco; 1 g $liter^{-1}$ each) and kept under a H_2-CO_2 gas mixture (80:20, vol/vol) at 50-kPa headspace pressure. The medium was reduced by adding a palladium catalyst (56) and adjusted to pH 7.0 with 1 M $NaHCO_3$. For cultivation at pH 10.0, $NaHCO_3$ was replaced by Na_2CO_3 and the concentrations of Ca^{2+}, Mg^{2+}, and PO_4^{3-} were reduced to 0.06, 0.05, and 0.14 g $liter^{-1}$, respectively, to avoid precipitation; the pH of the medium was adjusted by addition of 1 M Na_2CO_3.

The medium for aerobic cultivation (MM-5) was identical to medium AM-5 except that $NaHCO_3$ was replaced by sodium phosphate buffer (pH 7.0; final concentration, 20 mM; autoclaved separately), the concentrations of $MgCl_2 \cdot 6H_2O$ and $CaCl_2 \cdot 2H_2O$ were decreased to 0.1 and 0.015 g $liter^{-1}$, respectively, and trace element solution SL 10 was replaced by trace element solution SL 11 (29). Medium for aerobic cultivation also received less yeast extract and Casamino Acids (0.5 g $liter^{-1}$ each).

Substrates were added from sterile stock solutions before the medium was dispensed into the dilution tubes. Substrate concentrations for aerobic cultures were 2.5 mM (D-glucose) and 5 mM (L-lactate and aromatic compounds). For anaerobic cultures, the amount of substrate was doubled. All cultures were incubated at 30°C in the dark; the tubes were slanted and gently agitated on a rotary shaker (100 rpm).

Metabolic product profiles. Growth was routinely ascertained by checking turbidity and testing substrate utilization and product formation by HPLC (56). Inoculated tubes were scored as positive if substrate degradation was confirmed by the results of HPLC analysis of the culture supernatant after 2 weeks (oxic) or 4 weeks (anoxic) of incubation. The presence of sulfate-reducing bacteria and methanogenic archaea was inferred from the production of sulfide (25) or methane (47). The presence of homoacetogens was indicated when acetate production was well in excess over the maximum amount theoretically formed by substrate oxidation. Utilization of aromatic compounds was analyzed by HPLC (13). To ensure that all major products were accounted for, electron balances were routinely determined for each tube (56).

Statistics. Unless mentioned otherwise, each data set represents the mean (±standard error) of the results obtained with at least four different animals. Significance was evaluated by one-way analysis of variance ($P < 0.05$).

RESULTS

Total cell counts. The intestinal tract of *P. ephippiata* larvae consists of two major compartments—a long, cylindrical midgut decorated with three rings of midgut ceca, and a bulbous hindgut (Fig. 1A to D). Both gut compartments are colonized by prokaryotic microorganisms, with cell densities of $(8.9 \pm 3.5) \times 10^9$ cells per g (fresh weight) in the midgut and $(4.0 \pm 1.4) \times 10^{10}$ cells per g (fresh weight) in the hindgut (DAPI counts; second instar). Cell densities obtained for the third instar were in the same range; none of the values differed significantly due to the large individual variance. In all larvae, however, the hindgut values were significantly higher (three- to sixfold) than those for the midgut. Phase-contrast microscopy revealed the presence of cocci, rods, and filamentous morphotypes. Numerous ciliate protozoa were present in the hindgut but not in the midgut. Also nematodes were regularly encountered in the hindgut; in third instars, they reached a length of almost 10 mm, and up to 20 to 30 individuals per larva were observed.

Axial profiles of intestinal pH and redox potential. Microsensor measurements performed with intact intestinal tracts incubated in aerated Ringer's solution revealed that the guts of all instars were characterized by large dynamics of pH and redox potential (Fig. 1E and F). The intestinal pH increased sharply in the anterior midgut and reached maxima between pH 10.1 and 10.7 beyond the second crown of midgut ceca (Fig. 1E). The maximal values did not differ significantly among the instars (pH 10.2 ± 0.1, $n = 13$). Beyond the third crown of ceca, the pH declined again, and it remained quite constant at slightly alkaline values over the bulk of the hindgut (pH 8.4 ± 0.1, $n = 13$). Only in larvae of the second and third instars did the gut content return to neutral towards the rectum.

In contrast to the intestinal pH, the redox potential (E_h) of the midgut contents shifted considerably during larval development. The midgut of first-instar larvae was characterized by oxidizing conditions, whereas the E_h shifted to reducing conditions in larvae of the second and third instars (Fig. 1F). The contents of the anterior hindgut paunch showed reducing conditions in all instars ($E_h = -100 \pm 11$ mV, $n = 11$). In the posterior hindgut, the E_h shifted again to more positive values, especially in the smaller guts of the earlier instars.

Oxygen concentration profiles revealed steep gradients around the midgut and hindgut compartments; in no case did O_2 penetrate deeper than 100 μm into the lumen of the respective segments. The complete consumption of O_2 in the gut periphery led to anoxic conditions at the gut center of all instars (details not shown). Only in the small guts of the first instar did oxygen penetrate the thin tubular gut regions posterior to the third crown of ceca and posterior to the paunch (Fig. 1D). Hydrogen concentration profiles showed that the

TABLE 1. Methane production by the three instars of *P. ephippiata* larvae and by isolated midguts and hindguts (third instar) incubated separately in Ringer's solution under air

Larva or gut section	CH_4 production rate (μmol g^{-1} h^{-1})[a]	*n*
Larvae		
First instar	0.13 ± 0.04	4
Second instar	0.12 ± 0.03	4
Third instar	0.36 ± 0.11	6
Isolated gut sections		
Midgut	ND[b]	14
Hindgut	0.030 ± 0.007	14

[a] All values are based on the fresh weight of the whole animal and are means ± standard errors for *n* different larvae.
[b] ND, not detectable (<0.1 nmol g^{-1} h^{-1}).

accumulation of H_2 (approximately 60 to 70 Pa) was restricted to only a small region in the dorsal part of the hindgut paunch (Fig. 1D; details not shown), which is in direct contact with the posterior midgut in situ (Fig. 1C).

Gas exchange rates of larvae. Oxygen consumption rates of all instars were in the same range (~10 μmol g^{-1} h^{-1}), with a large individual variance. This might have been caused by differences in the activities of the individual larvae or by different developmental phases within an instar and was not further investigated. Also methane emission of individual larvae varied strongly (Table 1), with rates ranging between 0.05 and 0.45 μmol g^{-1} h^{-1}. In spite of all individual variance, however, the specific rates of methane formation were usually higher in the third instar.

Separate incubation of isolated midguts and hindguts of third instars revealed that CH_4 was produced exclusively by the hindgut (0.20 μmol g of hindgut^{-1} h^{-1}), which corresponds to less than 10% of the rate observed with living animals (Table 1). However, when isolated hindguts were incubated in the presence of potential methanogenic substrates, CH_4 formation was strongly stimulated (up to fivefold) over the endogenous rates. Interestingly, the stimulating effect of H_2 added to the headspace gas was much less pronounced than that caused by addition of methanol or formate to the incubation buffer (Table 2).

Microbial fermentation products. The intestinal tracts of all instars contained short-chain fatty acids and other metabolites typical of microbial fermentations. The total concentration of fermentation products in midgut and hindgut was significantly lower in the first instar than in the second and third instars (Table 3). The latter did not differ significantly from each other or from the total concentration of fermentation products in the hemolymph of the third instar (5.7 ± 1.2 mM [n = 5]). In all instars, midgut and hindgut contained high concentrations of inorganic carbon (ΣCO_2). While concentrations did not differ significantly among the instars, the concentrations in the midgut were significantly higher than those in the hindgut (Table 3).

However, the spectrum of fermentation products differed considerably between midgut and hindgut, and also among the different instars (Fig. 2), which indicated changes in the composition of the intestinal microbiota during larval development. High concentrations of lactate and acetate were present in the midgut of all larvae, with acetate predominating in the first instar and lactate in the later instars. Also formate accumulated in the midgut of all larvae, with highest concentrations in the third instar. By contrast, acetate was the most abundant fermentation product present in the hindguts of all larvae and was accompanied by relatively high concentrations of propionate in all instars except the first. Interestingly, the composition of the hemolymph (determined only for the third instar) reflected the spectrum of microbial fermentation products in the midgut rather than that in the hindgut (Fig. 2).

Metabolic product profiles in serial dilutions. In order to identify the metabolic potential of the gut microbiota and the relative abundance of major metabolic groups, enrichment cultures on different substrates were inoculated with 10-fold serial dilutions of midgut and hindgut homogenates of a *P. ephippiata* larva (third instar) amended with various substrates. Growth was recorded, and metabolic product profiles were determined for all dilutions.

Under oxic conditions, all substrates were completely oxidized to CO_2, whereas microbial fermentation products accumulated under anoxic conditions. Figure 3 illustrates the results obtained for a dilution series with glucose as substrate. A comparison of the product profiles obtained with midgut and hindgut homogenates incubated at pH 7 indicates that the microbiota of the two gut sections differed strongly. For the midgut, the formation of propionate and butyrate was observed only in the lower dilutions, whereas the products of the higher dilutions consisted almost exclusively of lactate, acetate, ethanol, and formate. For the hindgut, however, butyrate formation was observed even in the highest positive dilution, and also propionate was a major product in all except the highest positive dilution. Moreover, the number of positive tubes was usually one tube higher than in the case of the midgut.

When midgut homogenates were serially diluted in alkaline medium (pH 10), the number of the highest positive dilution was always identical to that obtained with glucose at pH 7. While the higher dilutions had product profiles similar to those of the corresponding series in neutral medium (Fig. 3), no butyrate was formed and propionate formation was reduced in the lower dilutions; also growth was slower than under neutral conditions. Similar results were also obtained with Casamino Acids (details not shown). The same dilution depth was obtained under oxic conditions.

Table 4 summarizes the results obtained with dilution series on other substrates; the results allows an estimation of the

TABLE 2. Stimulation of methane emission by isolated hindguts of *P. ephippiata* (third instar) incubated in Ringer's solution by the addition of external electron donors

Substrate added	CH_4 formation rate (μmol g^{-1} h^{-1})[a]		Stimulation (fold)[a]	*n*
	Basal	After addition		
H_2[b]	0.13 ± 0.03	0.21 ± 0.01	1.8 ± 0.4	3
Methanol[c]	0.15 ± 0.08	0.50 ± 0.20	4.3 ± 1.2	5
Formate[d]	0.28 ± 0.19	1.04 ± 0.50	4.9 ± 1.2	4

[a] All values are based on the fresh weight of the hindgut and are means ± standard errors for *n* different larvae.
[b] Headspace partial pressure of 20 kPa.
[c] Final concentration of 5 mM.
[d] Final concentration of 10 mM.

TABLE 3. Gut volume and total concentrations of fermentation products (converted to glucose equivalents) and dissolved inorganic carbon (ΣCO_2) in midgut and hindgut of the three instars of *P. ephippiata* larvae[a]

Larval instar	Vol (μl)			Fermentation products[b] (mM)			ΣCO_2[c] (mM)		
	Midgut	Hindgut	*n*	Midgut	Hindgut	*n*	Midgut	Hindgut	*n*
1	18 ± 1	14 ± 1	14	2.6 ± 0.6	1.4 ± 0.2	5	41 ± 11	27 ± 7	5
2	71 ± 9	74 ± 7	17	7.0 ± 2.7	6.2 ± 1.9	6	32 ± 8	24 ± 4	5
3	543 ± 43	398 ± 26	23	6.8 ± 1.3	7.5 ± 3.2	11	46 ± 8	23 ± 2	5

[a] Values are means ± standard errors for *n* different larvae.
[b] Based on the number of reducing equivalents released upon formal oxidation to CO_2.
[c] Total dissolved inorganic carbon (CO_2 plus HCO_3^- plus CO_3^{2-}).

abundance of the major metabolic groups in midgut and hindgut. The highest positive dilutions were consistently obtained for the hindgut compartment, both with glucose and with Casamino Acids as substrate, regardless of whether oxic or anoxic media were used for the enumeration. The apparent number of cellulose-degrading bacteria was inferred from the complete dissolution of filter paper disks incubated in the basal medium. It was 1 to 2 orders of magnitude lower than that obtained for bacteria degrading glucose or Casamino Acids, both in midgut and in hindgut. Cellulose degradation was observed only in oxic dilution series, and cellulose was not degraded under alkaline conditions.

Anaerobic bacteria were present both in midgut and in hindgut, although the apparent numbers were consistently higher in the hindgut (Table 4), where both lactogenic and butyrogenic physiotypes were present in the highest dilutions. Lactate-fermenting bacteria formed propionate and acetate as major products, and judging by the occurrence of propionate formation in the dilutions series on glucose, it appeared as if the same populations are also responsible for propionate formation in these cultures. Also methanogenic archaea seem to be present in similar abundance, whereas the apparent numbers of CO_2-reducing homoacetogenic bacteria and of lactate-oxidizing sulfate-reducing bacteria are considerably lower. Methane production was much lower in the alkaline series, whereas the apparent number of homoacetogens was unaffected by the pH of the medium. Aerobic bacteria mineralizing vanillate and cinnamate were present in considerable numbers, but aromatic compounds were not degraded under anoxic conditions; only demethylation of vanillate and side chain reduction of cinnamate occurred in lower dilutions.

DISCUSSION

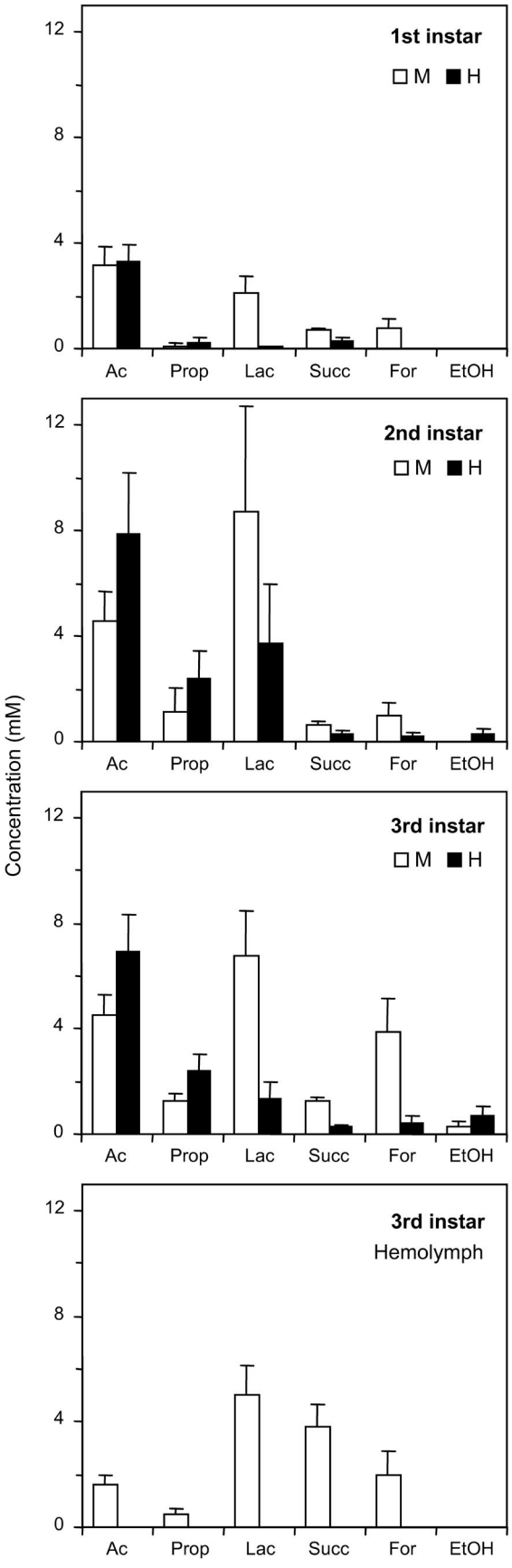
The importance of the gut microbiota in fiber digestion by scarabaeid beetle larvae was first recognized by Werner (59), who demonstrated cellulose degradation, proteolytic activities, and microbial fermentation products in the gut of the larva of *Potosia cuprea*. Later investigations revealed that the presence of a strongly alkaline midgut equipped with hydrolytic enzyme activities and a circumneutral hindgut characterized by microbial fermentation processes is typical for the larvae of the Scarabaeideae (3, 4, 30, 50, 60).

Physicochemical gut conditions. The first reports on the extremely alkaline pH in the midgut of scarabaeid beetle larvae date back almost 80 years (52, 58, 60). Grayson (30) compared the gut pHs of different coleopteran larva and found highest pH values in the midgut contents of *Osmoderma scabra* (pH 10.2) and *Cotnis nitida* (pH 10.1), which feed on decaying logs or humic soil; he carefully concluded that "those species feeding on decaying organic matter have a medium to strong alkaline reaction in the midgut."

The fine-scale intestinal pH profiles presented in this study document a pronounced dynamic of physicochemical conditions along the gut of *P. ephippiata*. The results are in good agreement with those reported for third-instar larvae of *Costelytra zealandica* (5), where the pH increased strongly in the prececal portion, remained rather constant (pH 10.8 to 10.9) in the anterior and middle portion, and dropped at the posterior end of the midgut, whereas a constant pH of 8.2 was present in the large hindgut. A similar situation seems to be present also in *Oryctes nasicornis*, where the midgut has a pH of 11.7 and the pH in the hindgut is 8.5 (3). The mechanism responsible for the generation of the alkaline pH remains to be investigated; it may be similar to that described for certain lepidopteran larvae (26, 32).

Although the midgut of *P. ephippiata* is mostly anoxic, most likely owing to the combination of microbial respiration and chemical oxygen consumption of the alkaline gut contents, as in soil-feeding termites (37), and highly alkaline, it has a relatively positive redox potential, whereas the neutral hindgut is more reduced. The E_h values are similar to those reported for the larva of *Oryctes nasicornis* (+30 mV in the alkaline midgut and −80 to −100 mV in the neutral hindgut [3]). Slightly lower redox potentials have been reported for midgut and hindgut of *Pachnoda marginata* (−100 to −200 mV [19]). The situation in humivorous beetle larvae is similar to that in soil-feeding termites (*Cubitermes* spp.), where the intestinal redox potential appears to be controlled not only by the presence of oxygen or hydrogen and the prevailing pH in the respective gut compartments but also by other redox-active components, such as humic substances and iron minerals, which are potential mediators and/or electron acceptors for the mineralization of organic matter (38). Interestingly, the conditions in the gut of the two smaller instars of *P. ephippiata* were slightly more oxidizing than those in the large gut of the third instar. This effect is probably related to the larger surface-to-volume ratio in the small instars, which should increase the rate of oxygen influx via the gut epithelium (11). Oxygen reduction in the gut periphery has been shown to affect the metabolic processes in the gut of *Reticulitermes flavipes* (55) and would also explain the differences in the fermentation product patterns between the instars of *P. ephippiata* (Fig. 2).

Hydrolysis of polymers. Many scarabaeid beetle larvae can feed exclusively on humus, e.g., on a peat soil devoid of living



plant roots. An analysis of the gut contents has revealed that larvae of *Adoryphorus couloni* feed preferentially on the organic soil constituents, which are sequestered at two to four times their concentration in the bulk soil (43). In *P. ephippiata*, the weight of the gut represents almost half the larval biomass (51, 44, and 38% in the first, second, and third instars, respectively), and based on dry weight, captive larvae produce almost two gut equivalents of feces per day.

The high pH in the midgut should facilitate the desorption of humic substances from the mineral matrix, thus rendering organic components accessible to enzymatic digestion (37).The black-brown contents of the alkaline midguts of *Cetonia aurata* and *Potosia cuprea* (pH 11 to 11.5) contain alkaline protease and amylase activities, whereas cellulase activities seem to be restricted to the hindgut (pH 7.0) (52, 58). In their investigations of *Oryctes nasicornis*, Bayon and Mathelin (4) have shown high rates of cellulose hydrolysis in midgut and hindgut, and also *P. marginata* larvae possess high activities of hydrolytic enzymes (xylanase and carboxymethyl cellulase) in midgut and hindgut (20). High protease activity has been reported for the midgut of third instars of *Costelytra zealandica* (5), and also the midgut of *Melolontha melolontha* larvae contains a complex mixture of proteinases, recently described in detail (57). Numerous alkali-stable proteases are also found in *P. ephippiata* (H. Zhang and A. Brune, unpublished results), and high ammonium concentrations in the hindgut of *P. ephippiata* (T. Lemke, X. Li, and A. Brune, unpublished results) indicate that the products of enzymatic hydrolysis are eventually subject to degradation by the animal and/or its gut microbiota.

Microbial fermentations. The exact contribution of the gut microbiota to the hydrolysis of different dietary components remains to be clarified since both the host and its microbial symbionts are potential sources of digestive enzymes. Nevertheless, the participation of gut microorganisms in the fermentative breakdown of the products of enzymatic hydrolysis is clearly evidenced by the high concentrations of microbial fermentation products in the midgut and hindgut fluid. The relative abundance of fermentation products in midgut and hindgut of *P. ephippiata* differs considerably and also changes during larval development. A dominance of lactate and acetate among the fermentation products in midgut and hindgut has been reported for *P. marginata* (20, 21), whereas acetate and propionate are the major metabolites in *Oryctes nasicornis* (3, 4).

As in other insects possessing a digestive microbiota, the microbial fermentation products will eventually be oxidized by the animal (10). This is in agreement with the presence of short-chain fatty acids in the hemolymph, which has been reported also for the larva of *Oryctes nasicornis* and *Popillia japonica* (3, 54). Although the pattern of metabolites in the hemolymph resembles that of the midgut more closely than

FIG. 2. Microbial fermentation products in midgut (M) and hindgut (H) of the three larval instars and in the hemolymph (third instar) of *P. ephippiata*. Concentrations were calculated using the geometrically estimated volumes. Bars represent standard errors for five separate guts ($n = 11$ for the third instar). Abbreviations: Ac, acetate; Prop, propionate; Lac, lactate; Succ, succinate; For, formate; EtOH, ethanol.

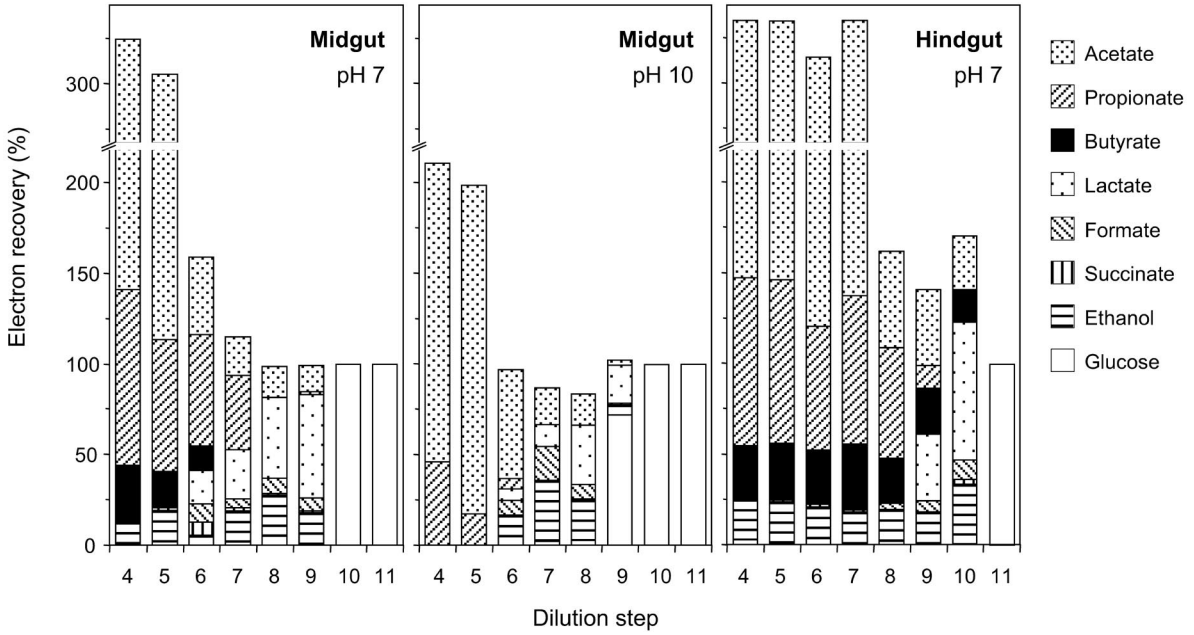


FIG. 3. Metabolic product profiles of enrichment cultures on glucose inoculated with 10-fold serial dilutions of midgut and hindgut homogenates of a *P. ephippiata* larva (third instar), incubated at pH 7 or 10. A dilution step of n is equivalent to 10^{-n} gut equivalents in the inoculum.

that of the hindgut, it has been pointed out already by several authors that the structure of the hindgut epithelium indicates that the proctodeal dilation is a zone of absorption also (2, 50, 60).

Major metabolic groups of microorganisms. The bacterial density in the two major gut compartments of *P. ephippiata* is similar to that reported for *P. marginata* (21). Little is known, however, about the microorganisms colonizing the intestinal tract of scarab beetle larvae and their metabolic activities.

TABLE 4. Abundance of major metabolic groups in serial dilutions of midgut and hindgut homogenates of *P. ephippiata* larvae (third instar)[a]

Metabolic group	Substrate	Midgut	Hindgut
Aerobic[b]	Cellulose[c]	8	7
	Glucose	9–10	10
	Casamino Acids[d]	9	10
	Vanillin, cinnamate	6–7	8
Fermenting[e]	Cellulose[c]	<4[f]	<4
	Glucose	9	10
	Casamino Acids[d]	8	9
	Lactate	7–8	8–9
Methanogenic[e]	H_2-CO_2	6–8	8–10
Homoacetogenic[e]	H_2-CO_2	4–5	7–8
Sulfate reducing[e]	Lactate, formate	4	6

[a] Values represent $\log_{10}$ of the last positive dilution in three to four replicate series at pH 7.
[b] Medium MM-5 incubated under air.
[c] Filter paper disks (equivalent to 5 mM glucose).
[d] Concentration was increased to 0.5 mg liter^{-1}.
[e] Medium AM-5 under H_2-CO_2 atmosphere.
[f] Dilution series started with the 10^4 dilution.

The cultivation-independent study published in the companion paper (28) showed that the microbial community in the midgut is composed almost exclusively of gram-positive bacteria, with clones associated with the *Actinobacteria*, the *Clostridiales*, and, to a lesser extent, the *Lactobacillales* and *Bacillales* dominating the clone library. Interestingly, several clones were virtually identical to the 16S rRNA sequence of *Promicromonospora pachnodae* isolated from the gut of *P. marginata* (23), which is closely related to the cellulolytic bacterium *Cellulosimicrobium variabile* isolated from the hindgut of the termite *Mastotermes darwiniensis* (1). Its presence in large numbers also in the gut of *P. ephippiata* larvae (O. Geissinger and A. Brune, unpublished results) suggests that *P. pachnodae* may be a specific member of the gut microbiota of *Pachnoda* larvae. *P. pachnodae*, initially designated "*Cellulomonas pachnodae*" (22), is a facultatively anaerobic bacterium that possesses endoglucanase and xylanase activity and ferments glucose to acetate, lactate, ethanol, and formate (23), which is in good agreement with the metabolic product pattern of the serial dilutions (Fig. 3).

The microbial community composition in the hindgut of *P. ephippiata*, however, differs markedly from that in the midgut (28). The clone library was dominated by clones associated with the *Cytophaga-Flavobacterium-Bacteroides* phylum, the *Clostridiales*, and the *Lactobacillales*; clones related to *Bacillales* and *Actinobacteria* were of minor frequency. Interestingly, the first bacterium ever isolated from scarab beetle larvae was a "*Bacillus cellulosam fermentans*" (59)—a gram-negative, obligately anaerobic, spore-forming bacterium that did not grow on monosaccharides, disaccharides, or starch, resembling Omelianski's "Wasserstoffbacillus" (45) and Khouvine's "*Bacillus cellulosae dissolvens*" (39) in the strong production of hydrogen and acidic fermentation products. The isolate was eventually

lost, but from the description and its association with cellulose fibers (59), it can be safely concluded that it was an obligate anaerobe, possibly representing a cellulolytic member of the *Cytophaga-Flavobacterium-Bacteroides* phylum or the clostridia.

Representatives of both groups seem to be numerically important in the gut of *P. ephippiata* (28). The presence of clostridia and *Bacteroides*-related bacteria is in good agreement with the formation of butyrate as a characteristic product in the highest dilutions of hindgut contents. Since butyrate production—at least in clostridia—is dependent on the hydrogen partial pressure in the environment (51), the apparent absence of butyrate accumulation in the hindgut fluid may be explained by the low H_2 partial pressure maintained in all hindgut regions.

Microorganisms capable of aerobic and fermentative metabolism appear to be similar in abundance or may even represent populations of facultatively anaerobic bacteria. Only the bacteria mineralizing vanillate and cinnamate seem to be obligately dependent on the influx of oxygen into the gut. Just as in the case of the termite gut microbiota (17), such lignin-derived aromatic compounds were not degraded under anoxic conditions; only demethylation of vanillate and side chain reduction of cinnamate occurred in the lower dilutions.

Methanogenesis. In *P. ephippiata*, methanogenesis takes place exclusively in the hindgut paunch but not in the alkaline midgut. This is in good agreement with the results of the cultivation-independent characterization of the archaeal community in the different gut compartments, which revealed that the dominant archaea in the midgut were (most likely nonmethanogenic) *Crenarchaeota* (28). Methanogenesis is restricted to the hindgut also in the larvae of other scarabaeid beetles (3, 31), and the methane emission rates are in the same range as those determined for *P. ephippiata* (Table 1). The large individual variance could be at least partly attributable to scarabaeid beetle larvae emitting CH_4 not by flatulence but by spiracular-controlled 6-min bursts only once every 1.5 h, as demonstrated for *Pachnoda butana* (6).

Similar to the situation in cockroaches and soil-feeding termites (42, 53), the methane emission rates of isolated hindguts of *P. ephippiata* were considerably lower than those of whole larvae, and methanogenesis was stimulated considerably by exogenous electron donors. In cockroaches (*Blaberus* sp.), a cross-epithelial transfer of H_2 from the midgut compartment drives methanogenesis in the hindgut (42), and also the high in vivo rates of methanogenesis in soil-feeding termites (*Cubitermes* spp.) have been explained by the proximity of H_2-producing and H_2-consuming gut compartments within the abdomen (53). However, in scarab beetle larvae, the discrepancy between methane emission rates of whole animals and isolated hindguts, which is also evident from the data for *Oryctes nasicornis* (3), cannot be explained by the same mechanism. H_2 does not accumulate in the midgut of *P. ephippiata*, and since the bulk of midgut and hindgut is not in direct contact in scarab beetle larvae (Fig. 1C), the highly diffusible H_2 molecule would not be a good transport form of reducing equivalents. Rather, the accumulation of formate as a major product of fermentation in the midgut, its presence in the hemolymph, and the strong stimulatory effect of formate on hindgut methanogenesis indicate that midgut fermentations are coupled to methanogenesis in the hindgut by formate transported via the hemolymph.

Conclusion. The digestive tract of humus-feeding scarab beetle larvae shows considerable parallels to the situation in soil-feeding termites, particularly with respect to the alkaline gut regions. The gut of *P. ephippiata* larvae harbors a dense and diverse microbiota, which differs considerably among the major gut regions and from that in the soil fed to the larvae (28). To date, only a few of these bacteria have been isolated in pure culture, but the results of the present study are quite encouraging for further cultivation-based investigations, which are important to improve our understanding of the functional interactions of the symbiotic microbiota involved in the digestion of soil organic matter by humivorous insects.

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